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Mac25 is the first member of the IGFBP-rP family (IGFBP-rP1). IGFBP-rP1 is expressed in normal human mammary epithelial cells (HMECs) and upregulated in senescent HMECs. IGFBP-rP1 is not expressed in twelve/sixteen breast cancer cell lines and its expression is absent in estrogen receptor positive (ER$^+$) breast cancer cell lines.

IGFBP-rP1 may regulate HMEC growth. We tested the hypothesis that it suppresses growth in ER$^+$ breast cancer cells by transducing the cDNA into normal and human breast tumor cultures. We will determine if IGFBP-rP1 is regulated at the transcriptional level by characterization of the promoter. We have determined binding properties of IGFBP-rP1 by growth assays in presence of IGF-I, IGF-II, R^3-IGF-I, and R^6-IGF-II and immunoprecipitation/phosphatase assays. We have determined IGFBP-rP1 tissue specificity by mRNA *in situ* hybridization.

We have ascertained that IGFBP-rP1 negatively regulates growth in ER$^+$ breast cancer cells. IGFBP-rP1 blocks growth stimulation by insulin, EGF, and IGF-II. Experiments indicate IGFBP-rP1 binds IGF-II. IGFBP-rP1 generated in ER$^+$ breast cancer cells exhibit two bands of higher molecular weight compared to protein found in ER$^-$ breast cancer cells. We have isolated one clone from a genomic library screen that contains approximately 1kb of sequence 5' of the translation start site.

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Karen Swisshelm   9/17/98
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U.S. Army Medical Research & Material Command
Fort Detrick, Maryland
Presentation of Annual Report
September 18, 1998

Mac25 (IGFBP-RP-1), a new insulin-like growth factor binding protein and its role in breast cancer
Mentor: Karen Swisshelm, Ph.D.
Trainee: Heather-Marie P. Wilson

Attached cover letter, 1 copy, unnumbered

Table of Contents

Mentor: Karen Swisshelm, Ph.D.
Trainee: Heather-Marie P. Wilson

Mac25 (IGFBP-RP-1), a new insulin-like growth factor binding protein and its role in breast cancer

INTRODUCTION

Background

Cell growth is regulated through the interaction of autocrine, paracrine, and endocrine growth factors; their receptors, and molecules that modulate factor/receptor interaction. The insulin-like growth factor (IGF) system includes proteins that contribute to growth regulation of mammary epithelial cells and have been extensively studied in breast cancer (1-3). Peptide growth factors known to stimulate breast cancer cell proliferation include insulin, IGF-I, and IGF-II. IGF-I and IGF-II have a structure that is homologous to proinsulin and stimulate growth predominantly through the IGF-1 receptor. Insulin can interact with both insulin and IGF receptors to enhance proliferation.

Interactions between growth factors and compatible receptors are frequently regulated through additional molecules that enhance or block interactions between ligand and receptor. IGF system includes the insulin-like growth factor binding protein (IGFBP) family that functions to modulate interactions between IGFs and their receptors. There are currently six IGFBPs (IGFBPs 1-6) and four potential new members (IGFBP-rP1/IGFBP-7, CTGF, *nov*, and *cyr61*)(4). The four new members show low affinity binding to IGF-I and IGF-II when compared with the original six members. These later factors have been designated as Insulin-like growth factor binding protein-related binding proteins (IGFBP-rPs) 1-4. Due to the various names under which Mac25/IGFBP-rP1 has been published, it was decided at the 4th International Symposium for IGFs (Tokyo, October 1997) that this molecule be categorized as insulin-like growth factor binding protein related protein 1 (IGFBP-rP1) until further characterization had been done (5, 6). *Therefore, Mac25/IGFBP-rP1 is will be referred to as IGFBP-RP-1 in this review.*

Isolation and identification of mac25/IGFBP-RP-1

IGFBP-rP1 was first suggested to be a member of the IGFBP family after it was isolated in a plasmid subtraction library screen from a meningioma cell line (7). IGFBP-rP1 was found in a study designed to uncover potential tumor suppressor genes by differentially displaying genes overexpressed in normal human mammary epithelial cells (8-10). The gene was mapped to chromosome 4q12 by FISH which is adjacent to a region (4q13) that is a hotspot for cytogenetic deletion in primary breast cancer (11). A

tumor suppression function for IGFBP-rP1 was suggested in experiments wherein a
murine IGFBP-rP1 was transduced into a p53-deficient human osteosarcoma cell line
(Saos II). This resulted in growth inhibition to the same extent as the p53-deficient Saos
II cells transfected with p53 (12). The mouse IGFBP-rP1 homolog does not contain the
IGF binding region which indicates that IGFBP-rP1 growth suppression function is
independent of IGF-regulation.

The many hats of mac25/IGFBP-rP1

In addition to its potential as a more generalized growth inhibitor and an IGF-
binding protein, IGFBP-rP1 has demonstrated activities that appear to be tissue specific.
Sequence analysis indicate that IGFBP-rP1 is 98% identical and 100% similar to
prostacyclin-stimulating factor (PSF) and tumor-derived adhesion factor (TAF) indicating
that mac25/IGFBP-rP1/PSF/TAF is the same protein which has prostacyclin-stimulating
activity in human diploid fibroblasts and cell adhesion activity in human bladder
carcinoma (13-15). Studies using C57BL/6 mice have shown mac25/IGFBP-rP1 is
expressed in most mouse tissues, with expression levels differing between male and
female mice in spleen, liver, kidney, indicating that IGFBP-rP1 may be under hormonal
regulation (16).

Hypothesis/Purpose

IGFBP-rP1 is a gene with high homology to a hormone/growth factor binding
protein in the IGFBP family (IGFBP-1: 37% identical, 75% similar). We hypothesize
that it may suppress the growth in an ER+ breast cancer cell line. Retinoids and estrogen
may transcriptionally regulate IGFBP-rP1.

Scope/Technical Objectives

To test the hypotheses presented above, we propose the following studies.

Specific Aims

Aim 1. We will test the potential of IGFBP-rP1 *function* as a negative effector of cellular
growth rates in ER+ breast cancer cells. We propose to transduce the cDNA into normal
and human breast tumor cultures using a retroviral vector system.

Aim 2. We will test the hypothesis that IGFBP-rP1 is *regulated* at the transcriptional
level by retinoic acid through a retinoic acid response element and by estrogen through an
estrogen response element via cognate receptors. We propose to characterize the IGFBP-
rP1 promoter.

Aim 3. We will determine protein binding *characteristics* of IGFBP-rP1 using purified
IGFBP-rP1 protein and a specific antibody.

Aim 4 (Additional). We will determine tissue specificity of RNA expression and protein localization by in situ hybridization using tissues collected from C57BL/6 mice. We began this last aim in order to determine if IGFBP-rP1 was *hormonally* regulated in a variety of tissues, with the mouse providing the easiest accessibility.

BODY

Aim 1: Experiments to address the potential of IGFBP-rP1 for modulating cell growth of ER+ breast cancer cells.

Experimental Methods

Previous Work/Background (August 1996-August 1997)
The full length IGFBP-rP1 cDNA has been cloned from a normal HMEC λ Zap cDNA library and inserted in the BamHI site of an expression vector containing a cytomegalovirus (CMV) promoter and the gene for the selectable marker for neomycin (vector from B.Vogelstein, unpublished) (8).

A retroviral vector system with the pLXSN vector (17-19) was used in transfection experiments studying the effect of IGFBP-rP1 in breast cancer cells lacking endogenous expression. The IGFBP-rP1 cDNA was transferred from the pCMV-neo vector to pBluescriptIIKS BamHI site, which allows for easier manipulation in cDNA amplification and sequence analysis. After sequence verification, the full-length IGFBP-rP1 cDNA was ligated into the retroviral vector, LXSN. This system relies on use of retrovirus-packaging cell lines, in the absence of helper virus, to aid in producing replication-defective retroviral vectors. The LXSN vector contains two promoters that are responsible for transcription of the inserted gene and the neomycin gene. In addition to the two promoters, there are long terminal repeats (L), a cloning site (X), a simian virus 40 early promoter (S, for transcription of the neomycin resistance gene), and the neomycin gene (N). The vector also has a ψ region that is required for packaging viral RNA into virions and allow the retroviral vectors to obtain higher colony forming unit (CFU) titers. Retroviral vectors containing sense and antisense orientations were created.

Ecotropic PE501 cells were transfected with LXSN, L*IGFBP-rP1*SN, and L*antiIGFBP-rP1*SN by calcium phosphate coprecipitaion. Virus was harvested and used to infect PA317 cells (amphotropic). Cells were selected using G418 and resulting clones were assessed for: unrearranged vector, vector titer, absence of helper virus, and expression of inserted gene.

Virus was collected from PA317 medium and used to infect Hs578T and MCF7 breast cancer cell lines. Clones were isolated, tested for IGFBP-rP1 expression (Northern), and stored at −70°C (liquid nitrogen) until use.

7

August 1997-August 1998

The focus of this year's studies has been to clarify the potential growth regulatory function of IGFBP-rP1 using MCF7 cells (no endogenous IGFBP-rP1 expression). MCF7 cells were transduced with LXSN/*LIGFBP-RP-1*SN and individual clones were isolated. To obtain a culture that was more representative of heterogeneous breast cancer *in vivo*, MCF7 cell were also mass transduced. Clones and mass cultures were evaluated growth kinetics, cell cycle, apoptosis, and senescence (Wilson, H-M.P. *et al.*, submitted, see addendum).

Results and Discussion

Growth curves revealed a 26-43% reduction of growth in mass transduced L*IGFBP-rP1*SN MCF7A breast cancer cells over a seven-day growth period in comparison to MCF7A LXSN. Single clone analysis demonstrated a 76-82% reduction in growth.

Cell morphology was altered in the MCF7 cells transduced with L*IGFBP-rP1*SN. MCF7 LXSN cells (control) were compact and uniform in size and appearance. MCF7 cells containing L*IGFBP-rP1*SN contained a significant population that exhibited enlarged cytoplasm, reminescent of senescent HMEC morphology. Moreover, the transduced cells presented multinucleation and multiple vacuoles.

Flow cytometry analysis demonstrated a higher percentage of the MCF7 L*IGFBP-rP1*SN cells in G1 compared to MCF7 LXSN. Flow cytometry and nuclear fragmentation analysis rule out apoptosis as the method for the observed cell reduction. The SA-β-gal assay showed the majority (80%) of MCF7 L*IGFBP-rP1*SN clonal cells positive for β-gal activity compared to MCF7 LXSN cells that were marginally positive (2%).

Metabolic assays comparing the responses between MCF7 LXSN and MCF7 L*IGFBP-rP1*SN cells using medium with SFM, serum-free medium (SFM), SFM with insulin (0.1nM final concentration), EGF in SFM (10ng/ml final concentration), IGF-I in SFM (10ng/ml final concentration), IGF-II in SFM (10ng/ml final concentration), R^3-IGF-I in SFM (10ng/ml final concentration), and R^6-IGF-II in SFM (10ng/ml final concentration). The MCF7 cells containing L*IGFBP-rP1*SN display a senescent-like response to all the growth factors, except the IGF-I and the long IGFs that have low or no affinity for IGFBPs (R^3-IGF-I and R^6-IGF-II), indicating that IGFBP-rP1 is preventing MCF7 growth stimulation through at least two pathways.

Conclusions/Ongoing Hypotheses

- IGFBP-rP1 inhibits/decreases growth of MCF7 breast cancer cells.
- IGFBP-rP1 alters cell morphology.
- IGFBP-rP1 blocks cell growth stimulation by insulin, EGF, and IGF-II.
- IGFBP-rP1 interacts directly with IGF-II to negatively regulate growth.

Aim 2: Experiments for promoter studies: library screening and clones analysis.

Experimental Methods

Previously, I screened a human chromosome 4 genomic library for clones that hybridized to IGFBP-rP1 and obtained ten clones. I analyzed these clones by restriction digest, PCR, and DNA-DNA hybridization of dot blots. Two clones (4 and 5) appeared to have inserts of approximately 5 kilobases that contained genomic DNA 5' of the IGFBP-rP1 gene. Two additional clones (1 and 9) may also contain additional genomic regions of IGFBP-rP1.

DNA from the four candidate clones were purified for further analysis by a λ DNA miniprep that relies on PEG precipitation/phenol:chloroform extractions from λ-bacteriophage-lysed XL-1 Blue *E.coli*. The purified double-stranded λ bacteriophage DNA was amplified by PCR using a primer for the 5' end of IGFBP-rP1 (nt 10-28, contains IGFBP-rP1 start codon region) and one of two primers that flank the insertion site of the λ bacteriophage. A fragment >1000 nucleotides was PCR-amplified from clone 5.

I am currently in the process of isolating the genomic DNA insert from purified λ bacteriophage and transferring it to pBluescriptII-KS so that it will be easier to obtain large amounts of purified plasmid DNA containing the genomic insert for sequence analysis and future experiments.

Results and Discussion

The focus of Aim 2 has been to try to isolate the promoter region. To do this, we have employed PCR amplification of the inserted using various primers to screen for the desired insert, an insert that contains region 5' of the start codon. After narrowing the field from ten to two clones, a primer specific for the 5' end of the gene was created and used to amplify upstream regions adjacent to the start codon. By using a primer that included the start codon, we increased the chance for the resulting amplified cDNA to include promoter region. At this point, a region >1000 nucleotides has been isolated. The next step will be to sequence this region and map it relative to the original clone.

The 300 bp region upstream of the transcription initiation site has been previously cloned (20). They found seven binding sites for Sp1. We manually examined this sequence and have identified what may be a novel retinoic acid response element (RARE). By isolating the promoter region, we will be able to confirm if this is indeed a new RARE or an error in sequencing. The original mac25/IGFBP-rP1 sequence submitted to Genbank contains an extra nucleotide at position 830 (Accession L19182, (7)). We discovered this inaccuracy when confirming our original cDNA sequence, which was supported by our results with RT-PCR/sequencing of four mammary cell lines. We plan to continue with our experiments to confirm the published promoter sequence, determine if we have additional 5' sequence of the promoter region, and to test functional characteristics of the promoter by luciferase assays.

- 1 clone identified: contains >1000 nucleotides of cDNA sequence 5' of the start codon and most likely contains the promoter region.
- 4 clones identified (including one described above): contain part/all of genomic IGFBP-rP1.

Aim 3: Experiments to determine binding characteristics of IGFBP-rP1 using purified IGFBP-rP1 protein and a specific antibody.

Experimental Methods

Conditioned SFM was collected from Hs578T (control) and transduced MCF7 cells (described previously in Aim 1) and concentrated onto nitrocellulose membrane. The protein was eluted from the membrane by boiling 10 minutes in sample buffer containing 8M urea. Sample was loaded onto a 15% separating gel with a 4% stacking gel, run 30 minutes at 100 volts, increased to 185 volts for two hours, transferred to PVDF membrane by wet transfer using transfer buffer containing SDS for 1 hour at 100 volts. The membrane was then blocked in 0.5% milk/1% BSA in 0.05% Tween-20 Tris Buffered Saline (TTBS). The membrane was incubated at 1:2500 dilution of the primary antibody (anti-hRIGFBP-rP1 (21)) overnight at 4°C with shaking. The membrane was washed 3X for 15 minutes each in TTBS and incubated with secondary antibody (Anti-rabbit HPR from donkey, Amersham) at 1:3000 for two-three hours at room temperature. Membrane was washed in TTBS for three 15 minutes washes. Pierce Super Signal Chemiluminscence was used to detect the secondary antibody.

Results and Discussion

By Western blot analysis, we were able to detect expression of the IGFBP-rP1 protein in MCF7 cells transduced with L*IGFBP-rP1*SN. It is interesting to note that the protein expressed in the MCF7 breast cancer cells has two forms of a higher molecular weight than the endogenous protein expressed in Hs578T breast cancer cells. Computer analysis of the sequence revealed the following motifs for potential post-translational modification: four casein kinase phosphorylation sites, five protein kinase C phosphorylation sites, nine N-myristoylation sites, and one N-glycosylation site.

- 31 kDa IGFBP-rP1 protein detected in Hs578T (positive control)
- Two forms of IGFBP-rP1 protein (~33 and ~34 kDa) detected in cells transduced with MCF7 (31kDa seen in only one clone that also contained the modified protein).

Aim 4 (Addition): Experiments to determine if tissue specificity is gender dependent for RNA expression/protein localization of IGFBP-rP1 in mice.

<u>*Results and Discussion*</u>

Results from Aim 4 were presented in Tokyo, Japan October 21-24, 1997 at the 4th International Symposium on Insulin-like Growth Factors (abstract, in addendum)(16).

- Tissue distribution: IGFBP-rP1 RNA expressed in all tissues tested.
- Higher expression in female kidney and liver when compared to male.
- Cell specific hybridization occurring in white pulp region of female spleen.
- Female cell specific expression observed in epithelial cells that line the lumen of the uterine horn and ductal epithelium, throughout ovary, and in ductal epithelium of mammary gland.

CONCLUSION

Function of IGFBP-rP1

Cell growth assays using LXSN and L*IGFBP-rP1*SN transduced MCF7 breast cancer cells that do not express endogenous IGFBP-rP1 demonstrate the antiproliferative activities of IGFBP-rP1 *in vitro*. Expression of IGFBP-rP1 induced a senescent phenotype observed by morphological changes and senescence-associated β-gal activity. Apoptosis was not the mechanism by which cell growth was reduced in cells expressing IGFBP-rP1, as determined by flow cytometry and nuclear fragmentation assays.

Posttranslation modification of IGFBP-rP1

IGFBP-rP1 protein appears to have been posttranslationally modified when expressed in MCF7 cells when compared to the endogenous IGFBP-rP1 expressed by Hs578T. There are several motifs present in the IGFBP-rP1 sequence that would allow for phosphorylation, N-glycosylation, and/or N-myristoylation. Our next goal will be to elucidate the type of modification occurring in the MCF7 breast cancer cells.

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APPENDICES

- 4th International Symposium on Insulin-like Growth Factors, October 21-24, 1997.
  Mac25 (IGFBP-7) Expression in Mouse. H-M.P. Wilson and K.L. Swisshelm (16).

- *Introduction of mac25/IGFBP-rP1 into MCF7 breast cancer cells induces growth
  inhibition and senescent phenotype.* H-M.P. Wilson, R.S. Birnbaum, M. Poot, and
  K. Swisshelm. Department of Pathology, University of Washington School of
  Medicine, Seattle, WA, 98195 USA *(for submission)*.

Figures 1-4. Tissue was collected from ICR (outbred) and C57BL/6 (inbred) mouse
strains and snap frozen in O.C.T. freezing medium. ^{33}P-labeled sense (control) and
antisense riboprobes were generated using T7 and T3 polymerases and digested pBl-
IGFBP-rP1 (IGFBP-rP1 ligated into pBluescriptII-KS at BamHI site). Preparation of
tissues on slides were done following standard procedures (22).

Figure 1. Brain panoramic. *A.* Bright field, H&E, general brain structure and
orientation. *B.* Dark field, demonstrates IGFBP-rP1 mRNA expression.

Figure 2. Brain. Comparison of IGFBP-rP1 expression levels between male and female
C57BL/6 mice.

Figure 3. Uterus. IGFBP-rP1 expression is found in epithelial cells that line lumin of
uterine horn and ductal epithelial of C57BL6 female mouse.

Figure 4. Ovary and Mammary Tissue. IGFBP-rP1 is found throughout ovary and in
the ductal epithelium of mammary tissue.

Statement of Work

Technical Objective 1
Test the potential of IGFBP-rP1 function.

Task 1: (Months 1-4) Prepare plasmids for transfection. Ligate full-length IGFBP-rP1 cDNA into retroviral vector. *Completed*

Task 2: (Months 5-12) Transfection of packaging cell lines and transduction of breast cancer cell lines, select clones and begin to test growth parameters. *Completed*

Task 3: (Months 15-24) Complete growth studies, using specific growth factors. *Completed – paper for submission*

Technical Objective 2
Characterize the mac25 promoter.

Task 1: (Months 4-7) Finish screening genomic libraries for IGFBP-rP1 promoter. *Completed*

Task 2: (Months 12-15, 24-28) Restriction and sequence analysis of promoter clones. *In Progress*

Technical Objective 3
Determine protein binding characteristics of mac25.

Task 1: (Months 1-12) Test antiserum generated mac25 peptide by Elisa. Have access to an antibody generated in another lab that has been successfully used in our lab. *Completed*

Task 2: (Months 12-28) Perform immunoblots for normal HMECs and breast tumor cell lines. *In Progress*

Task 3: (Months 15-24) Determine ligand interaction with IGFBP-rP1. *Completed*

Technical Objective 4 (Additional)
Determine IGFBP-rP1 tissue specificity of RNA.

Task 1: (Months 5-12) Test mRNA expression localization in various mouse tissue. *Completed – abstract presented*

Task 2: (Months 24-30) Determine RNA expression and protein localization in normal breast and tumor tissue. *In Progress*
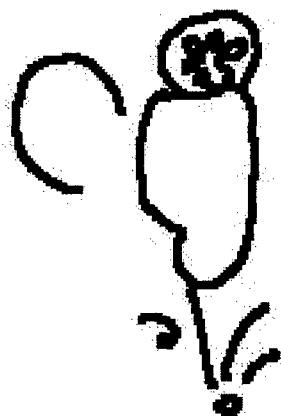
Figure 1

BRAIN

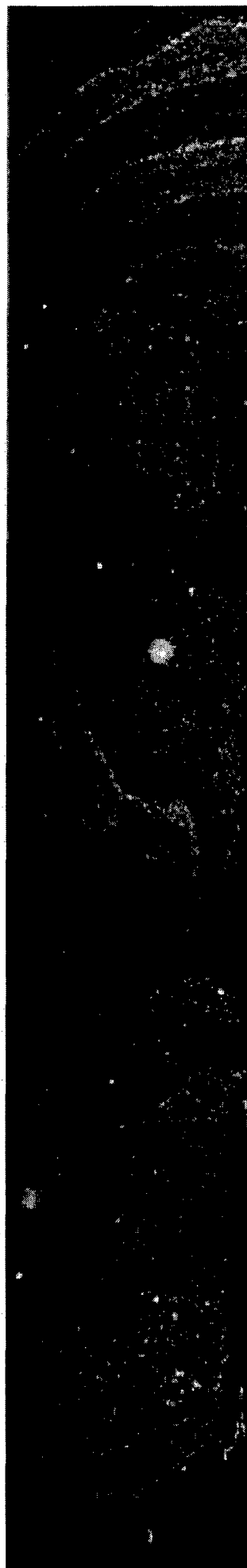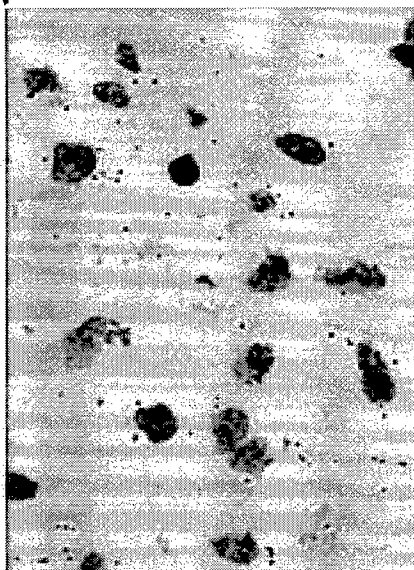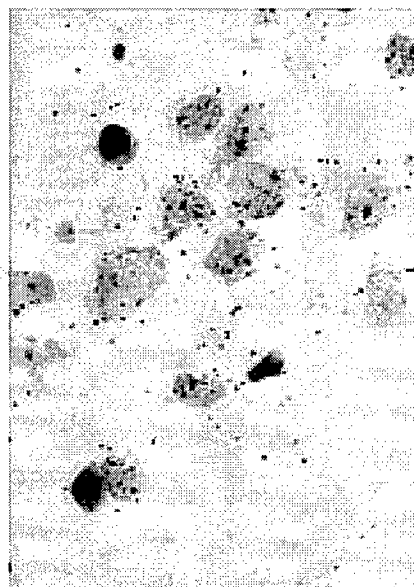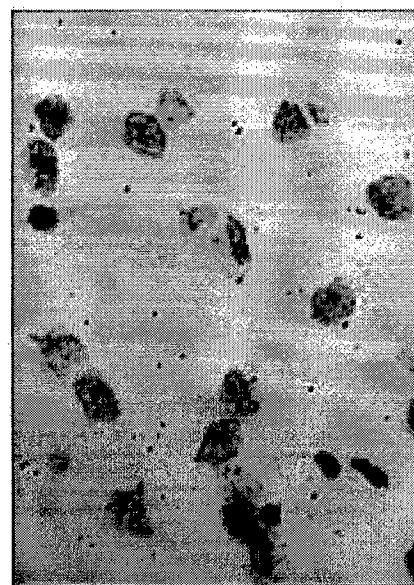**A. Female brain, bright field**

B. Female brain, dark field
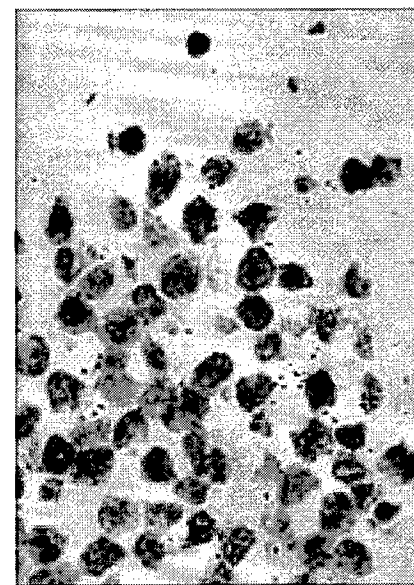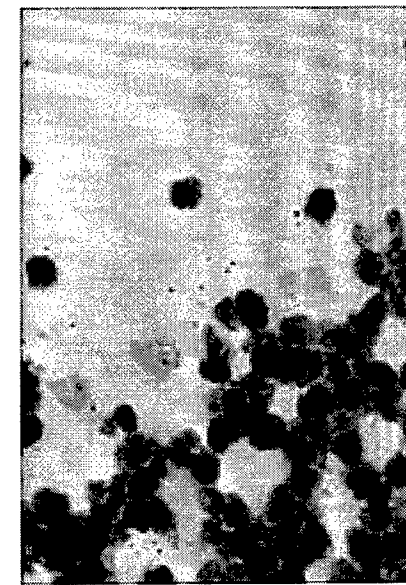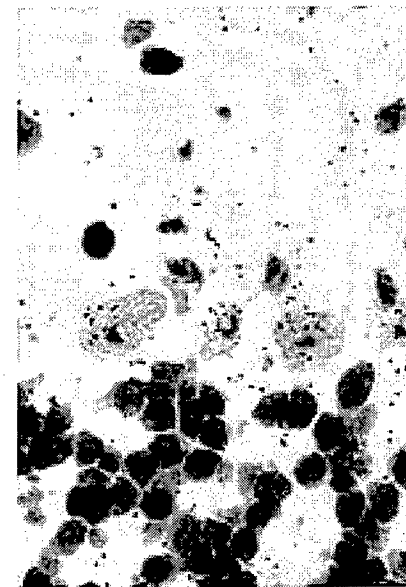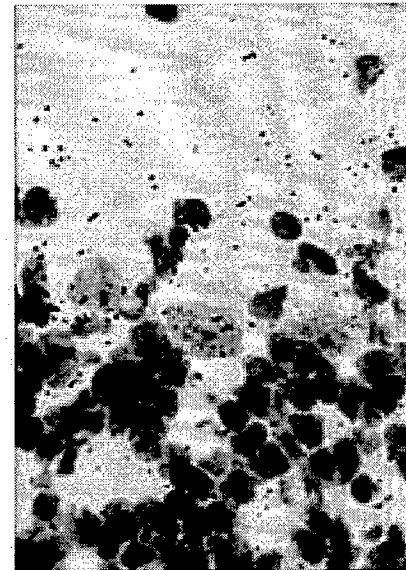
Figure 2

ANTISENSE, MALE

ANTISENSE, FEMALE

SENSE, FEMALE

CORTEX

HIPPOCAMPUS

CEREBELLUM

17

Figure 3

UTERUS

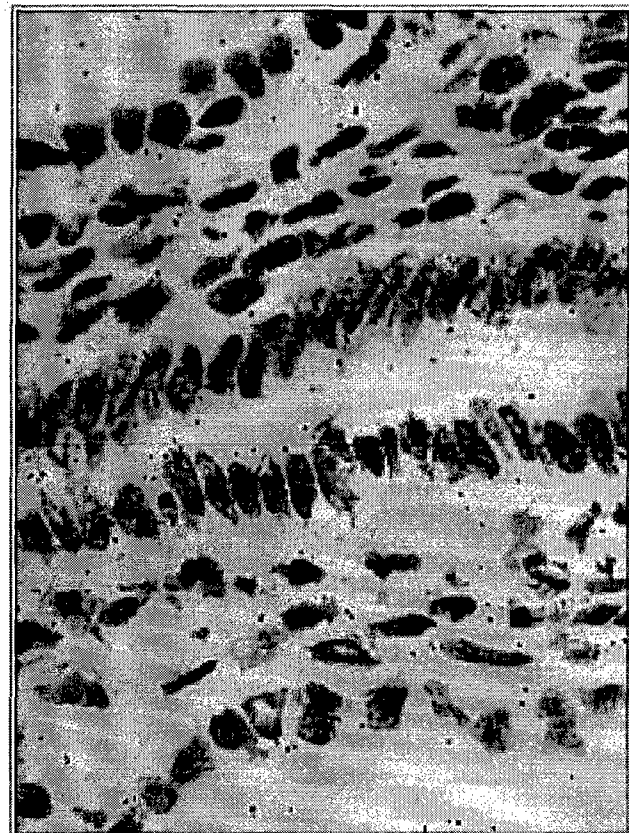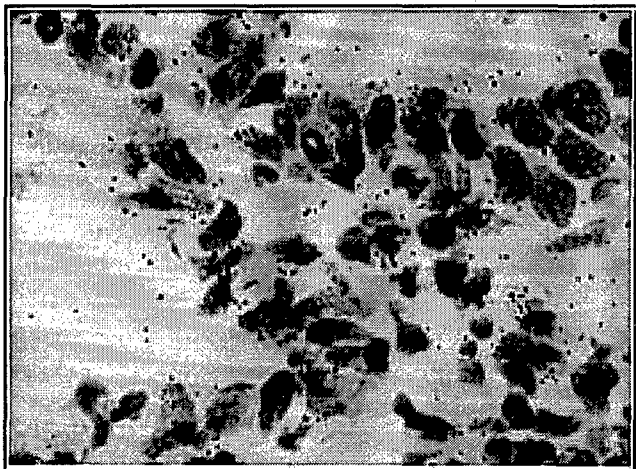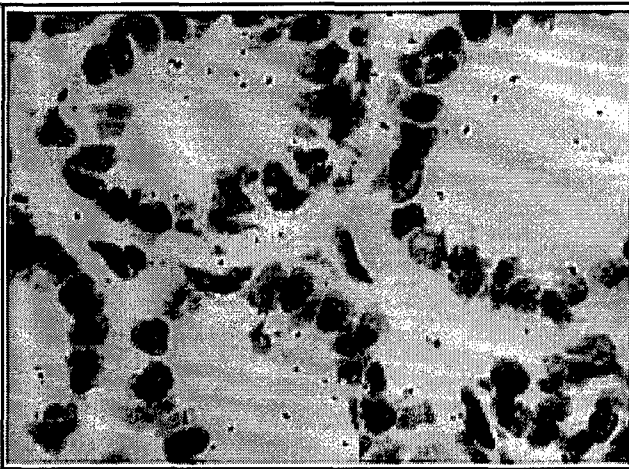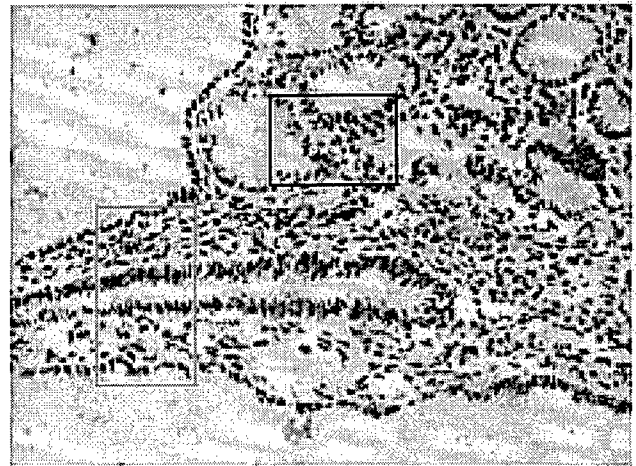**SENSE**   **ANTISENSE**
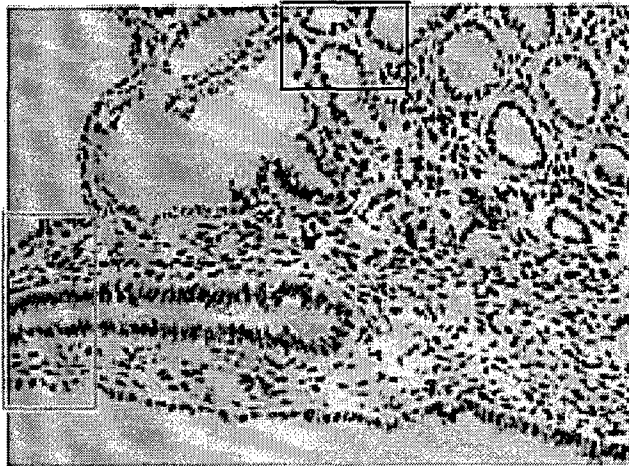
Figure 4

OVARY
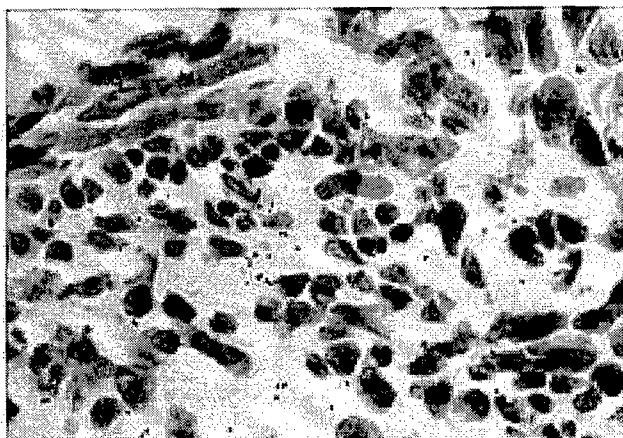
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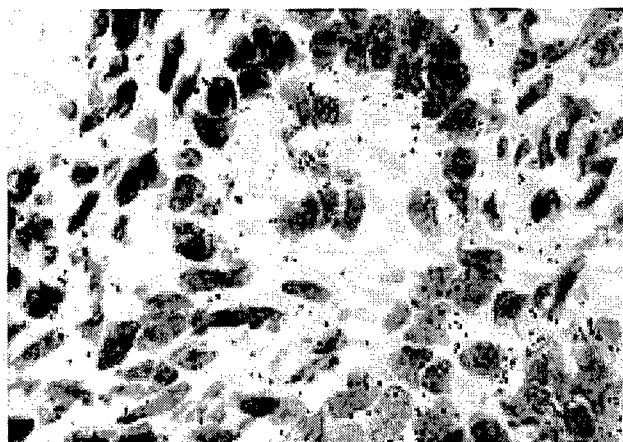
MAMMARY TISSUE
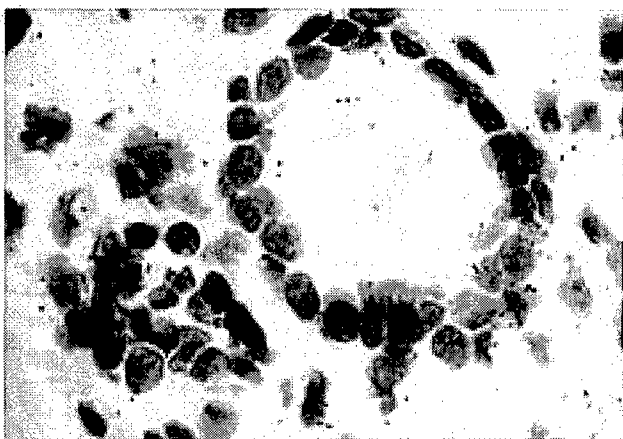
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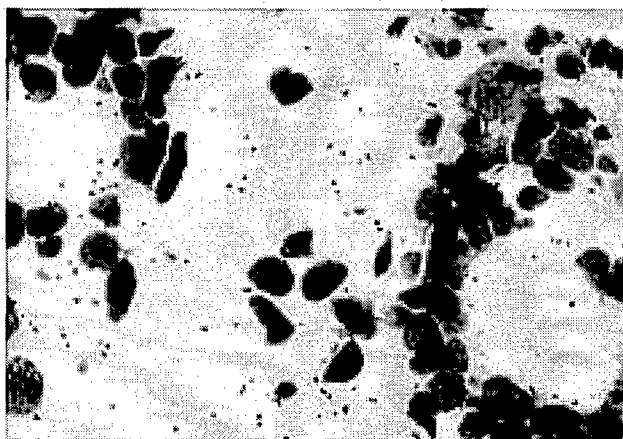
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Introduction of mac25/IGFBP-rP1 into MCF7 breast cancer cells induces growth inhibition and senescent phenotype.

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Running Title:
Mac25/IGFBP-rP1 in MCF7 breast cancer cells.

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Abstract:

Insulin growth factor binding proteins (IGFBPs) modulate growth of cultured human mammary epithelial cells (HMECs) by regulating the availability of insulin-like growth factors (IGFs) to target cells. IGF binding proteins with lower affinity for IGFs are members of a related family, the IGFBP-rPs. Our studies with the IGFBP-rP1 gene (mac25/TAF/PSF) suggest an additional role for the protein as IGFBP-rP1 expression is up-regulated in senescent HMECs. We introduced the IGFBP-rP1 cDNA by retroviral transduction into MCF-7 breast cancer cells, which lack detectable endogenous IGFBP-rP1 expression. We observed a reduction in the population doubling of approximately 80% in MCF-7 cells transduced with IGFBP-rP1 compared to cells transduced with the LXSN vector alone or the parental line. Moreover, cell cycle analyses revealed a decline in the percent of cells in S-phase of MCF-7 cells expressing IGFBP-rP1 and diminished response to growth factors, insulin, IGF-II, and EGF. IGFBP-rP1-positive MCF-7 cells exhibited an altered morphology, from a pleomorphic cobblestone shape to an enlarged and flattened shape similar to senescent HMECs. Consistent with the morphologic change, we detected pH6-dependent β-galactorsidase activity in 80% of the cells. These experiments suggest that IGFBP-rPs may constitute potent replicative inhibitory function in mammary epithelial cells.

Abbreviations used:

EGF, epidermal growth factor; HMECs, human mammary epithelial cells; IGF, insulin-like growth factor; IGFBP, insulin-like growth factor binding protein; IGFBP-rP1, insulin-like growth factor binding protein related-protein 1; SA-β-gal, senescence associated β-galactosidase; SFM, serum-free medium.

INTRODUCTION

The IGF system and its role in regulation of breast tumor growth has been the focus for many studies (1). Members of this system include insulin, IGF-I, IGF-II, the IGFBP family and the corresponding receptors. Insulin, IGF-I, and IGF-II are known to stimulate breast cancer cell proliferation. IGF-I and IGF-II growth stimulation is mediated mostly through the IGF-I receptor. Insulin can interact with both insulin and IGF receptors to enhance proliferation (2). The insulin receptors are present in higher numbers in breast cancer cells in comparison to normal breast tissue (3, 4). Estrogens increase the expression of IGF-I receptor mRNA (5) and may be one pathway used by estrogen to stimulate growth of breast epithelial cells. Recent epidemiological studies on premenopausal breast cancer have shown in a correlation between increased risk and elevated insulin levels (6) as well as higher circulating IGF-I levels (7) suggesting that their increased presence may stimulate tumorigenesis.

Insulin, IGF-I, IGF-I, and relaxin are known members of the insulin family (8). To date, there are no known binding proteins for insulin or relaxin. However, a family of six insulin-like growth factor binding proteins (IGFBPs 1-6) with potentially four new members (mac25/IGFBP-rP1, CTGF, *nov*, and *cyr61*) are known to regulate the interactions of IGF-I and IGF-II with the IGF-1 and IGF-2/M-6-P receptors (1, 9-12). The four potential members are currently classified as IGFBP-related proteins (IGFBP-rPs) (13, 14). IGFs preferentially bind IGFBPs over the IGF receptors which allow these proteins to fulfill the role of IGF modulator.

IGFBP-rP1 was first isolated by differential expression between a normal leptomeningeal cell line and a meningioma cell line using a subtraction library screen (15). A subsequent protein homology search predicted the 102 amino acid sequence of this gene to be 37% identical and 75% similar to human IGFBP-1. IGFBP-rP1 was also identified in a screen for genes upregulated in senescent human mammary epithelial cells (HMECs) (16). It has been hypothesized that genes upregulated during senescence may also function as tumor suppressor genes (17). Further support for IGFBP-rP1 tumor or growth suppressing function was shown in a human p53-deficient osteosarcoma cell line (Saos II) transfected with *murine* IGFBP-rP1 that displayed growth inhibition similar to Saos II cells transfected with p53 (18). It is interesting to note that the mouse IGFBP-rP1 homolog lacks the IGF binding region, indicating that IGFBP-rP1 functions independently of IGF-binding to inhibit growth. Recent ligand blot studies demonstrate the ability of IGFBP-rP1 to bind insulin, IGF-I, and IGF-II at high concentrations (11, 12). Further studies are needed to assess physiologic binding.

In addition to being a potential growth inhibitor and potential IGF-binding protein, IGFBP-rP1 demonstrates tissue specific activities. Sequence analysis indicated IGFBP-rP1 to be 85% identical and 92% similar to human prostacyclin-stimulating factor (PSF) and human tumor-derived adhesion factor (TAF) (19). This indicates IGFBP-rP1/mac25/PSF/TAF is the same protein and that it has prostacyclin-stimulating activity in human diploid fibroblasts (20) and cell adhesion activity in human bladder carcinoma

(21). These studies indicate that IGFBP-rP1 plays multifunctional roles in various systems.

In this study, we transduced MCF7 human breast cancer cells with a retrovirus containing the full-length IGFBP-rP1 construct, demonstrating that IGFBP-rP1 inhibits growth and induces a senescent phenotype. Clones containing IGFBP-rP1 showed a decline in the percent of cells entering the S phase of the cell cycle and an increase in senescent-associated β-galactosidase activity. IGFBP-rP1 diminished cell growth/survival response to insulin, EGF, and IGF-II. IGFBP-rP1 had little, if any, inhibitory effect on IGF-I and no effect on long-IGFs (R^3-IGF-I and R^6-IGF-II). Our experiments indicate the IGFBP-rP1 regulates IGF-II by direct interaction *in vitro* and may be referred to as IGFBP-7.

MATERIAL AND METHODS

IGFBP-rP1 sequence verification.
The full-length construct cDNA of IGFBP-rP1 was cloned from a normal HMEC λ ZAP cDNA library and inserted into a pCMV-neo-BamHI vector (B.Vogelstein, unpublished vector) (16). The insert size of ~900 base pairs was confirmed by a BamHI digest and transferred to pBluescriptII-KS in the BamHI site (Stratagene). The IGFBP-rP1 construct was sequenced by automated and manual procedures, taking advantage of the T3 and T7 primer sites present in pBluescriptII-KS. Automated sequencing was done at University of Montana by Joan Strange. Double-stranded plasmid sequencing was performed by an adapted method (J. Oshima, unpublished) of the Sequenase Version 2.0 DNA Sequencing Kit (United States Biochemical, Cleveland, OH). Primers designed to corresponding regions within the IGFBP-rP1 cDNA sequence found in Genbank (15) (IGFBP-rP1 - 291; IGFBP-rP1 - 752; IGFBP-rP1 - 898; T3; T7) and T3/T7 primers to the promoter regions present in pBluescriptII-KS were used for sequencing.

Reverification of full-length mac25 cDNA sequence in four breast cell lines.
RNA from four cell lines AG11132, AG11134, Hs578T, and MBA-231 were reverse transcribed using M-MuLV according to the manufacturer's protocol (Beohringer, Germany). AG11132 and AG11134 were obtained from reduction mammoplasty and are considered to be normal. Primers were designed and used to amplify the region between nucleotides 752 and 898. PCR products were sequenced by using the same primers described above and an adaptation of the protocol above used for plasmid DNA using Sequenase (United States Biochemical, Cleveland, OH). 15 μl of the PCR reaction was treated with 3 μl shrimp alkaline phosphatase and 3 μl exonuclease I, incubated at 37°C for 15 minutes followed by 80°C for 20 minutes in a thermocycler to remove unincorporated primers and dNTPs. 1 μl of primer (100ng) was added to 9 μl of the treated PCR reaction, denatured at 100°C for 5 minutes, and placed in an ice/water bath for 5 minutes. 8 μl of the denatured/annealed sample was then sequenced as described above in Plasmid Sequencing.

Sequencing studies on pBluescriptII-KS IGFBP-rP1 (pBl-IGFBP-rP1) determined that the full-length IGFBP-rP1 construct contains 865 nucleotides (nucleotides 9-874 in

Genbank). There were six nucleotide deviations that resulted in six conservative amino acid changes from the sequence submitted to Genbank by Murphy et al. (22/23CG to GC, 44T to 44C, 50C to 50G, 291G to 291A, 297 G to 297A, and 584C to 584T) and one guanosine nucleotide "deletion" at position 830. The sequences obtained from four breast cell lines match the sequence found in the pBl-IGFBP-rP1 construct from the 76N library. The derived amino acid sequence is 98% identical and 100% similar to the sequences submitted to Genbank for tumor-derived adhesion factor (TAF) and prostacyclin-stimulating factor (PSF)(19).

Construction of IGFBP-rP1 retroviral vector and generation of retrovirus-producing cells.

IGFBP-rP1 cDNA was ligated into the BamHI site of the pLXSN plasmid (A. Dusty Miller, (22-24)), transformed into DH5 cells, and grown on LB plates containing ampicillin. Plasmid DNA was following a lysozyme-based small scale preparation found in Maniatis (25) using a modified resuspension buffer of 8%sucrose, 0.5% Triton X-100, 50mM EDTA, and 10 mM Tris-HCl, pH 8 (Gary Warren, unpublished). The plasmid was digested with SmaI to determine IGFBP-rP1 orientation in the vector (Boehringer Mannheim). The pL*IGFBP-rP1*SN was verified.

PE501 and PA317 retroviral-packaging cells were used to generate virus (A. Dusty Miller, (24). PE501 were transformed with the plasmid vector and selected with 1mg/ml G418 (Calbiochem), and was grown with 0.75mg/ml thereafter to ensure that selected cells contained the vector. Harvested virus from PE501 cells were used to infect PA317 packaging cells. Clonal vector-producing cells were isolated with G418 as described above and tested for unrearranged vector (Southern), vector titer, and expression of inserted plasmid and IGFBP-rP1 gene (Northern). Virus was harvested from PA317 clones that exhibited intact vector and demonstrated RNA expression of the inserted plasmid and high vector titers. MCF7 cells were transduced by LXSN or L*IGFBP-rP1*SN virus in the presence of 4 µg/ml polybrene. Individual clones were isolated following selection in 1mg/ml G418 and maintained in 0.75mg/ml G418. To obtain a culture that was more representative of what occurs *in vivo*, MCF7 cells were mass transduced to produce a heterogenous culture of many clones. In this paper, we assayed three clone pairs from MCF7 LXSN and L*IGFBP-rP1*SN, two pairs of mass transduced cultures, and the unaltered, parental MCF7 cells for changes in cell growth, cell cycle, and response to growth factors.

Southern and Northern Analyses.

MCF7 cells were grown to confluency on P100s and collected using Ultraspec-II RNA Isolation System (Biotecx Laboratories, Inc., Houston, TX). Northern and Southern analysis followed protocols previously described using full-length IGFBP-RP-1 cDNA as a probe (26).

Cell Culture.

PE501 and PA317 cells were grown in αMEM supplemented with 10% fetal bovine serum and 0.75mg/ml G418. MCF7A cells were grown in αMEM (Gibco BRL) containing HEPES (0.01M final concentration, Sigma), sodium pyruvate (1mM final concentration, Sigma), non-essential amino acids (1x final concentration, Sigma), EGF

(12.5ng/ml final concentration, Sigma), insulin (1µg/ml final concentration, Sigma), hydrocortisone (1µg/ml final concentration, Sigma), and 5% fetal bovine serum (Hyclone), (referred to as complete αMEM). Transduced cells containing retroviral vector were grown in complete αMEM supplemented with 0.75mg/ml G418 (Calbiochem). Serum-free medium (SFM) was made with αMEM supplemented with HEPES (0.01M final concentration), sodium pyruvate pyruvate (1mM final concentration), non-essential amino acids (1x final concentration), and 0.05% BSA (Sigma). In experiments requiring SFM, the cells were first washed with 1xPBS.

Immunoblotting.

Conditioned medium was collected and immediately concentrated to nitrocellulose with a dot blot apparatus. The concentrated protein was eluted in 25ul of 1x sample buffer of 0.5M Tris, 10% glycerol, 8M urea, and 2% SDS. Samples were boiled for 10 minutes, centrifuged briefly and 20 µl loaded onto a 15% polyacrylamide gel with a 4% polyacrylamide stacker. Gel was run at 100 volts for 30 minutes and 185 volts for 2 hours. Proteins were transferred to PVDF (Biorad) at 100 volts for 1 hour in transfer buffer containing 0.04% (w/v) SDS. The membrane was incubated with 10% hydrogen peroxide for 10 minutes. The membrane was blocked 1 hour in 1%BSA/0.5% blotting-grade nonfat dry milk in 0.05% Tween-20/Tris Buffered Saline (TTBS), washed with TTBS for three 15 minute incubations, incubated overnight at 4°C with the primary antibody, anti-hRIGFBP-rP1 (27) at 1:2500, washed for three 15 minute incubations, and incubated for 3 hours with anti-rabbit Ig horseradish peroxidase linked whole antibody from donkey at 1:3000 (Amersham Life Science). The membrane was treated with Supersignal Chemiluminescent Substrate (Pierce, Rockford, IL) for 5 minutes and exposed to CL-Xposure Film (Pierce).

Cell Growth Curves.

MCF7 cells containing LXSN and *LIGFBP-rP1*SN were plated on ten p60s at 50,000cells/p60 and grown in complete αMEM. Cell counts were taken for two plates on days 1, 2, 3, 5, and 7 using a hemocytometer. Cell numbers were statistically analyzed by student's t-test. This experiment was repeated two times each for two mass transduced MCF7A cell cultures. Triplicate growth curves were done on three clone pairs.

Flow cytometry.

Transduced MCF7A cells were plated in 6-well plates at a concentration of 21,600 cells/well in complete αMEM. On day 7, the cells were harvested by trypsinization. To avoid loss of cells, the conditioned medium, PBS wash, cells in trypsin, and the complete αMEM used to rinse the well were combined from each well into a 15ml screw cap tube and centrifuged >3,000g for 5 minutes. The supernatent was discarded and the resulting pellet was resuspended with one mL of medium that contained Hoechst 33342 (20 µM final concentration, Sigma) and 1µl CMXRos (200nM final concentration, Molecular Probes, Eugene, OR). Cell were incubated at 37°C for 30 minutes. After staining, 40,000 cells were analyzed using a Coulter Epics Elite Flow Cytometer (Coulter, Hialeah, FL) with time resolved dual 15 mW 488 nm argon (first) and 20 mW UV argon (delayed) excitation. The following fluorescence emissions were

collected: UV-excited blue fluorescence for Hoescht 33342; 488 nm excited CMXRos fluorescence with a 645 nm long pass filter. Data were analyzed using the MultiPlus (Multiparameter Flow Cytometry Data Analysis) package software (Phoenix Flow Systems, San Diego, CA). Apoptotic cells were identified as cells having lowered CMXRos fluorescence (28).

Nuclear fragmentation Assay.

MCF7A cells were plated in p60s at a concentration of 50,000 cells in complete αMEM. Cells were collected on day 4 as described in flow cytometry section (see above) and pelleted by centrifugation. Cell were resuspended and fixed in freshly prepared methanol:acetic acid (3:1) and placed at -20°C for a minimum of 24 hours. Fixed cells were repelleted, resuspended in freshly prepared methanol:acetic acid (3:1), and applied on glass slides cleaned with ethanol and allowed to air dry. Fixed cells were incubated in 2 µM Hoescht 33258 (in PBS) for 30 minutes at room temperature. Air dried and counted on a Zeiss fluorescent microscope using standard DAPI excitation and emission filters.

Cell Morphology.

Photomicrographs of cells were obtained on a Nikon Diaphot-TMD Inverted microscope using phase-contrast settings and Kodak Techpan film. Pictures were taken of day 7 plates before counting.

Senescence associated β-galactosidase (SA-β-gal) staining.

Cells plated at 50,000 cells/p60 in complete αMEM, cultured for 4 days, washed with PBS, and stained as described by Demri *et al.* (29). 500 cells per plate were counted.

Metabolic response to growth factors.

MCF7A cells were plated in six-well plates at a concentration of 205,000 cells/well in complete αMEM for 24 hours. Cells were wash once with PBS and replaced with serum-free medium (SFM) for another 24 hours. Cells were counted from two wells for each cell line on Day 0 to establish the start point. The following media was added to six wells; complete αMEM, SFM, insulin in SFM (1nM final concentration, Sigma, St. Louis, MO), EGF in SFM (10ng/ml final concentration, Sigma), IGF-I in SFM (10ng/ml final concentration, Bachem, Torrence, CA), and IGF-II in SFM (10ng/ml final concentration, Bachem). Duplicate cell counts (2 wells) occurred at 24, 48, and 96 hours after addition of SFM plus growth factor. These experiments were performed in triplicate. R^3-IGF-I (10ng/ml final concentration, GroPep, Adelaide, Australia) and R^6-IGF-II (10ng/ml final concentration, GroPep) were included in the second and third experiment in addition to the previously listed growth factors.

Statistical analysis.

Data collected were analyzed using the student's t-test. Flow cytometry results were analyzed using Mplus (Multiple Option Flow Cytometry Data Analysis package written by Peter S. Rabinovitch, Phoenix Flow Systems, San Diego) followed by the student's t-test.

RESULTS

Introduction of IGFBP-RP-1 into MCF7 breast cancer cells.

We chose to introduce IGFBP-rP1 into the MCF7 human breast cancer cells because they lacked expression of the gene and there has already been extensive studies of the IGF system in this cell line (30-33).

Northern analysis demonstrated the presence of IGFBP-rP1 expression in the MCF7A cells transduced with L*IGFBP-rP1*SN and its absence in LXSN control MCF7A cells **(Figure 2A)**. Western analysis demonstrated the presence of mac25 protein expression in the L*IGFBP-rP1*SN-transduced MCF7A cells **(Figure 2B)**. Two bands of approximately ~33 and 34 kDa were expressed in MCF7A cells compared to endogenous IGFBP-rP1 secreted from Hs578T human breast cancer cells (~31 kDa), suggestive of differential post-translational modification.

IGFBP-rP1 diminishes growth over seven day period in MCF7 breast cancer cells.

Growth curves show a range of 26-43% reduction of growth in mass transduced L*IGFBP-rP1*SN MCF7A cells over a seven-day growth period in comparison to MCF7A LXSN cells. Growth reduction in the range of 76-82% is observed in single clones transduced with IGFBP-rP1. **Figure 3** shows a representative graph from each cell line tested from a total of 13 growth curves: mass transduced cells T1 and T2 (in duplicate) and single clones 1, 2, 3 (in triplicate).

Expression of IGFBP-rP1 in MCF7 breast cancer cells induces morphologic alteration.

MCF7A control cells containing LXSN were more compact and uniform in size **(Figure 4)**. MCF7A L*IGFBP-rP1*SN cells showed a large number of cells with an increased cytoplasmic/nuclear ratio reminescent of senescent cell morphology. Many of these distended cells were multinucleated and contained multiple vacuoles. Moreover, MCF7A L*IGFBP-rP1*SN cells that were not in contact with other cells exhibited ruffling and more floating dead cells were observed when compared with MCF7 LXSN cells.

Presence of IGFBP-rP1 inhibits response of MCF7 cells to mitogens.

Comparison of cell growth response between MCF7 LXSN and MCF7 L*IGFBP-rP1*SN breast cancer cells demonstrated that insulin, EGF, IGF-I, or IGF-II in SFM were unable to stimulate growth in cells containing IGFBP-rP1 **(Figure 5)**. In contrast, MCF7 L*IGFBP-rP1*SN cells exhibited exponential growth in completed αMEM, although this growth was less than the MCF7 LXSN in SFM and under half the growth rate of MCF7 LXSN cells in completed αMEM. It is possible that the combination of growth factors and/or some unknown factor in the FBS is responsible for the MCF7 L*IGFBP-rP1*SN cells being able to proliferate. The higher growth rate of MCF7 LXSN in SFM could be due to autocrine growth stimulation in response to the absence of growth factors in the medium.

IGFBP-rP1 reduces number of cells entering S phase.

Four pairs were tested: one mass-transduced pair and three clone pairs **(Table 1)**. All L*IGFBP-rP1*SN MCF7 cells showed a higher percentage of G1 compared to LXSN MCF7. A decrease in MCF7 L*IGFBP-rP1*SN cell cycle S phase was detected in all four lines. A greater amount of cell debris was detected in all three clonal L*IGFBP-rP1*SN cell lines. Differences in apoptosis were not observed and similar results were obtained with the nuclear fragmentation assay **(Table 2)**.

IGFBP-rP1 diminishes response to growth factors.

Cell growth response to insulin, EGF, IGFs, and long IGFs were compared between MCF7 LXSN clone 1 and MCF7 L*IGFBP-rP1*SN clone 1 over a four-day period **(Figure 5)**. Day 0 was counted and set as 100% to which subsequent counts taken at 24, 48, and 96 hours were compared. Both clones showed high growth in complete αMEM (~300% increase). The MCF7 cells with LXSN showed an increase in cell numbers in response to all growth factors tested (EGF, +21%; IGF-I, +48%; IGF-II, +37%; R^3-IGF-I, 114%; R^6-IGF-II, 146%), except insulin which showed no growth (-3%) that was comparable with SFM (-9%). There was a loss in cell number in MCF7 cells containing IGFBP-rP1 in response to insulin (-62%), EGF (-18%), and IGF-II (-39%). There was a positive growth response to IGF-I (+37%) in the presence of IGFBP-rP1. There was an appreciable augmentation of growth in the presence of the long IGFs (R^3-IGF-I and R^6-IGF-II) that was comparable in MCF7 cells with and without IGFBP-rP1. The increased response to R^3-IGF-I in LXSN and L*IGFBP-rP1*SN MCF7 cells (+114% and +170%, respectively) can be explained by the long IGFs bypassing other regulatory IGFBPs present in MCF7 cells. The dramatic difference in growth response to IGF-II and R^6-IGF-II in MCF7 cells expressing IGFBP-rP1 protein indicates that one mechanism by which IGFBP-rP1 regulates growth is by directly binding to control IGF-II.

DISCUSSION

The IGF system has been under intensive scrutiny during past decade as a therapeutic target for breast cancer treatment. Several studies have shown correlation between high circulating IGF levels and increased risk, higher numbers of insulin and IGF receptors present in breast tumor cells, and increased expression of IGF-I and IGF-II in tumor cells (6, 7, 34, 35). Insulin, IGF-I, and IGF-II are proliferative agents that have repeatedly demonstrated an anti-apoptotic protective effect in breast tumor cells (36-39). These mitogens activate the insulin, IGF-I, and IGF-II receptors and are regulated by IGF binding proteins.

Due to the protein homology that IGFBP-rP1 shares with the members of the IGFBP family, it is very likely that it regulates a member of the insulin peptide superfamily. Previous studies have indicated that IGFBP-rP1 can achieve action similar to p53 by suppressing growth in a p53-deficient osteosarcoma cell line although the exact signaling pathway is unknown (18). IGFBP-rP1 is upregulated in senescent HMECs, which suggests of a role of the protein in growth suppression (16).

In seeking a functional role of IGFBP-rP1, we have introduced the gene into a

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human breast cancer cell line lacking IGFBP-rP1 expression. Growth curves allowed comparison in MCF7 cells transduced with LXSN vs MCF7 cells transduced with L*IGFBP-rP1*SN demonstrated that the presence of IGFBP-rP1 decreases cell proliferation. This revealed that MCF7 LXSN and MCF7 L*IGFBP-rP1*SN altered the phenotype and induced a change in cell morphology. The morphology of the MCF7 LXSN cells was unaltered, whereas the MCF7 L*IGFBP-rP1*SN cells showed a phenotype usually seen only in senescent HMECs, having an enlarged, flat appearance with vacuole formation around the nucleus. This suggested the possibility, as previously predicted by Swisshelm *et al.*, that IGFBP-rP1 is a factor associated with a senescent phenotype.

We wished to determine a mechanism for the increased population doubling time in the cells expressing IGFBP-rP1. Analysis by flow cytometry revealed that L*IGFBP-rP1*SN MCF7 displayed a decrease in percentage of cells in S phase indicating less proliferation is occurring in these cells. Apoptosis as the mechanism responsible for differences in cell growth was ruled out by flow cytometry and nuclear DNA fragmentation assay.

Growth suppression and senescent phenotype displayed by IGFBP-rP1 in MCF7 cells are indicative that IGFBP-rP1 causes cells to enter senescence. Therefore, we tested these cells to determine if their response to mitogenic agents such as insulin, EGF, IGF-I, or IGF-II is inhibited by IGFBP-rP1. The MCF7 cells containing IGFBP-rP1 showed a loss of cells to all the growth factors tested that are known to be growth stimulatory in breast cancer cells. One exception was IGF-I that induced an increase in cell growth response in MCF7 L*IGFBP*-rP1SN cells that was comparable to the response seen in MCF7 LXSN cells. This positive response could be due to the amount of IGF-I that was used (10ng/ml final concentration). The biological activity of IGF-I indicated to ranges between 0.2 to 20 ng/ml (Bachem, Analytical Data Sheet, H-5555) which was the range we tested. One group has indicated that the physiologic concentration of IGF-I is between 0.2 to 5 ng/ml and used 10 ng/ml IGF-I in their experiments (40). We also tested the response to R^3-IGF-I and R^6-IGF-II which have the same biological activity as IGF-I and IGF-II, but with low or no affinity for the binding proteins. MCF7 cells expressing IGFBP-rP1 showed a large increase in growth to the long IGFs when compared with native IGF response, indicating that IGFBP-rP1 may be controlling cell proliferation by more than one pathway. Because it appears that IGFBP-rP1 is negatively regulating the IGFs by direct interaction in *in vitro* studies, it is very likely that IGFBP-rP1 is IGFBP-7 and supports previous data from other groups indicating by ligand blot that IGFBP-rP1 binds IGFs (11, 12).

We tested the cells further by looking for senescence-associated β-gal (SA-β-gal) activity and discovered that 80% of the MCF7 cells containing IGFBP-rP1 were positive for SA-β-gal activity, whereas the MCF7 LXSN control cells had 2% SA-β-gal activity. It has been hypothesized that inhibitors of DNA synthesis are expressed by senescent cells that prevents them from entering S phase (41, 42). Senescent cells do not proliferate in response to physiologic mitogens and demonstrate changes in cell functions which may be reflected by the increased expression of SA-β-gal (29, 43). These traits that characterize senescence have been reflected in our studies with IGFBP-rP1 in MCF7 breast cancer cells.

Endogenous expression of IGFBP-rP1 is found in breast cancer cells that lack estrogen receptor expression (15, 16). A prominent question raised by our studies is why

IGFBP-rP1 does not decrease growth in these cells if we see attenuated proliferation in breast cancer cells that have estrogen receptor. In western analysis, Hs578T breast cancer cells exhibit endogenous protein of ~31 kDa. The transduced MCF7 cells expressed IGFBP-rP1 protein that was in two forms of higher molecular weight (~33 and ~34 kDa). Computer analysis of the amino acid sequence of IGFBP-rP1 indicates the following putitive sites: four casein kinase phosphorylation sites; five protein kinase C phosphorylation sites; nine N-myristoylation sites; one N-glycosylation site. It is possible that post-translational modification of one or more of these sites is necessary for IGFBP-rP1 to be metabolically active in regulating cell proliferation. The proteins responsible for modification of IGFBP-rP1 may be dependent upon estrogen and one of the receptors.

Experimental data implicates IGFBP-rP1 to be involved in growth regulation of breast cancer cells. The mechanism of replication regulation indicates that induction of a cellular senescence pathway is a possible scenario. Future studies will be necessary to further classify IGFBP-rP1 as a senescence factor, which includes determining if MCF7 Lmac25SN cells show a resistance to apoptotic stimuli, another hallmark of senescent cells. We will be determining what role IGFBP-rP1 may be performing in an *in vivo* system by studying the IGFBP-rP1 expression levels through immunocytohistochemistry and RNA in situ hybridization of human breast tissue. IGFBP-rP1 may be a useful marker in determining status of human breast tumors.

FIGURE LEGENDS

Figure 1. Retroviral vector constructs for transduction of MCF7 human breast cancer cells. A. Control LXSN vector, ~3.3 kb. B. L*IGFBP-rP1*SN vector, ~4.2 kb.

Figure 2. IGFBP-rP1 expression in transduced MCF7 breast cancer cells. A. Northern blot probed with full-length mac25 cDNA. B. Western blot incubated with anti-hrIGFBP-rP1 antibody (R. Rosenfeld) as described in Material and Methods.

Figure 3. IGFBP-rP1 suppresses growth in MCF7 breast cancer cells. *A.* Comparison of parental MCF7 and MCF7 LXSN clones demonstrate similar growth rates. *B.* Mass transduced MCF7 cell pairs of LXSN vs L*IGFBP-rP1*SN. (This graph contains two paired representatives that were each tested twice.) *C.* MCF7 paired clones of LXSN vs L*IGFBP-rP1*SN. (This graph contains three paired representatives from triplicate experiments.)

Figure 4. Senescent morphology observed in transduced L*IGFBP-rP1*SN MCF7 breast cancer cells. LXSN and L*IGFBP-rP1*SN were plated at 50,000 cells/p60 and counts taken on days 1, 2, 3, 5, and 7. Photomicrographs were taken on day 7 with a Nikon Diaphot-TMD Inverted microscope on phase-contrast setting and Kodak Techpan film.

Figure 5. IGFBP-rP1 abrogates response of MCF7 breast cancer cells to mitogens. Cells were grown as described in Material and Methods. At 0 hour, complete medium; SFM; and SFM with 0.1nM insulin; SFM with 10ng/ml EGF; SFM with 10ng/ml IGF-I; SFM with 10ng/ml IGF-II; SFM with 10ng/ml R^3-IGF-I; or SFM with 10ng/ml R^6IGF-II. Duplicate wells were counted at 24, 48, and 96 hours.
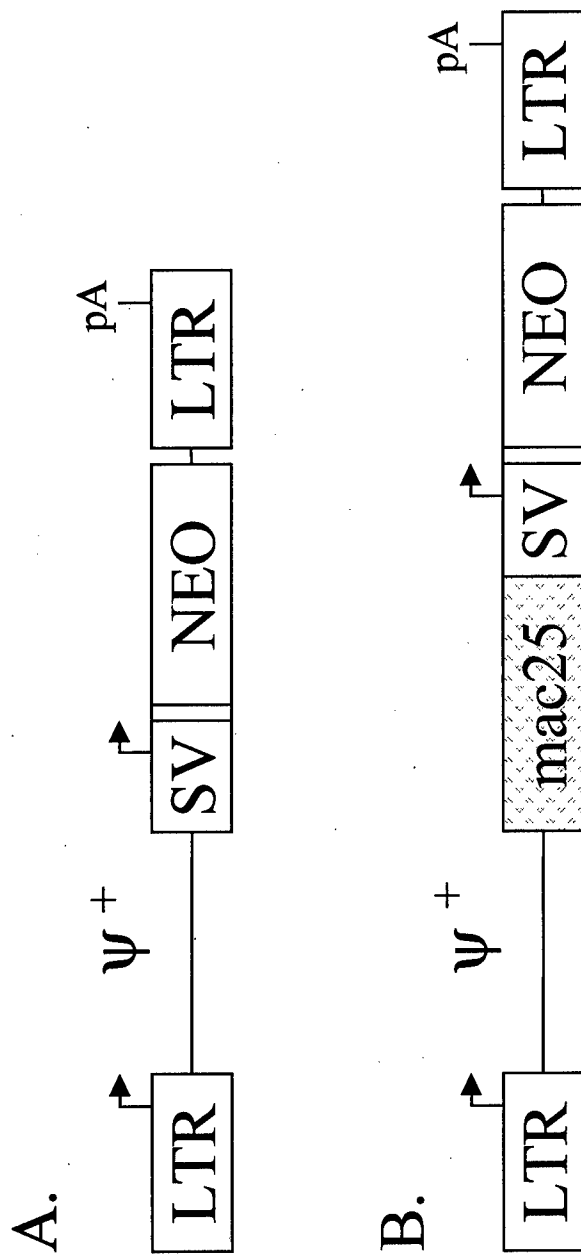
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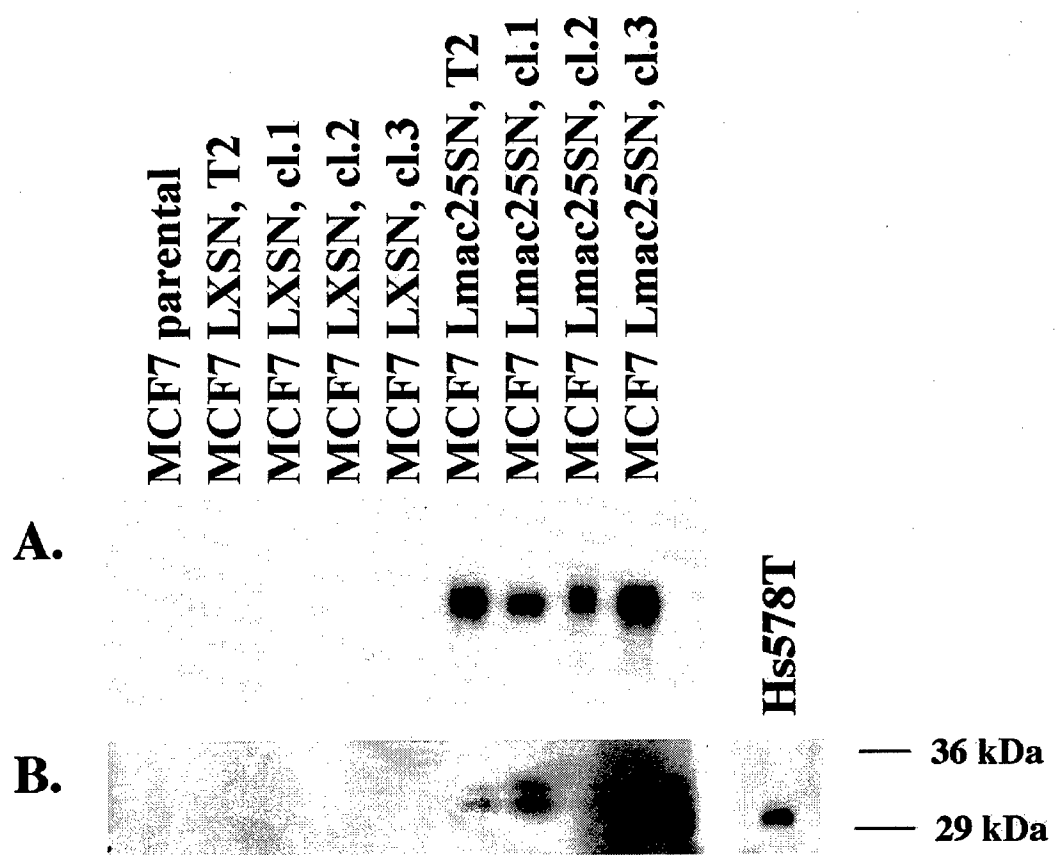
Figure 1.

A.

Ψ^+

LTR — SV | NEO | LTR — pA

B.

Ψ^+

LTR — mac25 | SV | NEO | LTR — pA

Figure 2.

Figure 3.

A.

Parental and LXSN MCF7

Cell Number(1,000s)

Days

- parental
- LXSN, cl1
- LXSN, cl2
- LXSN, cl3

B.

Mass Transduced MCF7 cells

Cell Number(1,000s)

Days

- LXSN,T1
- Ligfbp-rp-1SN,T1
- LXSN,T2
- Ligfbp-rp1SN,T2

C.

Transduced MCF7 clones

Cell Number(1,000s)

Days

- LXSN, cl1
- LXSN, cl2
- LXSN, cl3
- Ligfbp-rp1SN, cl1
- Ligfbp-rp 1SN, cl2
- Ligfbp-rp 1SN, cl3

Figure 4.

Figure 5.

LXSN, cl.1

LIGFBP-rP1 SN, cl.1
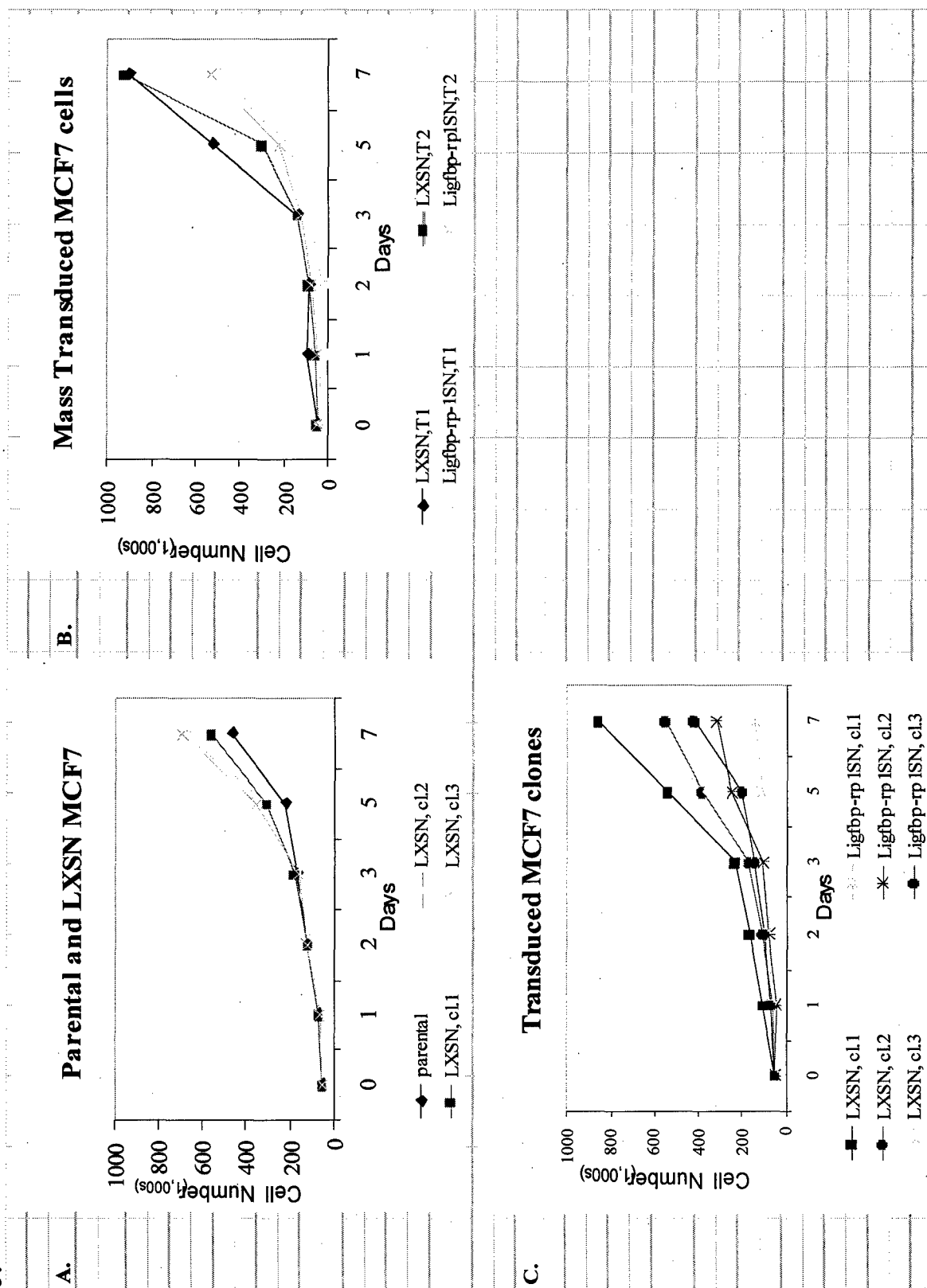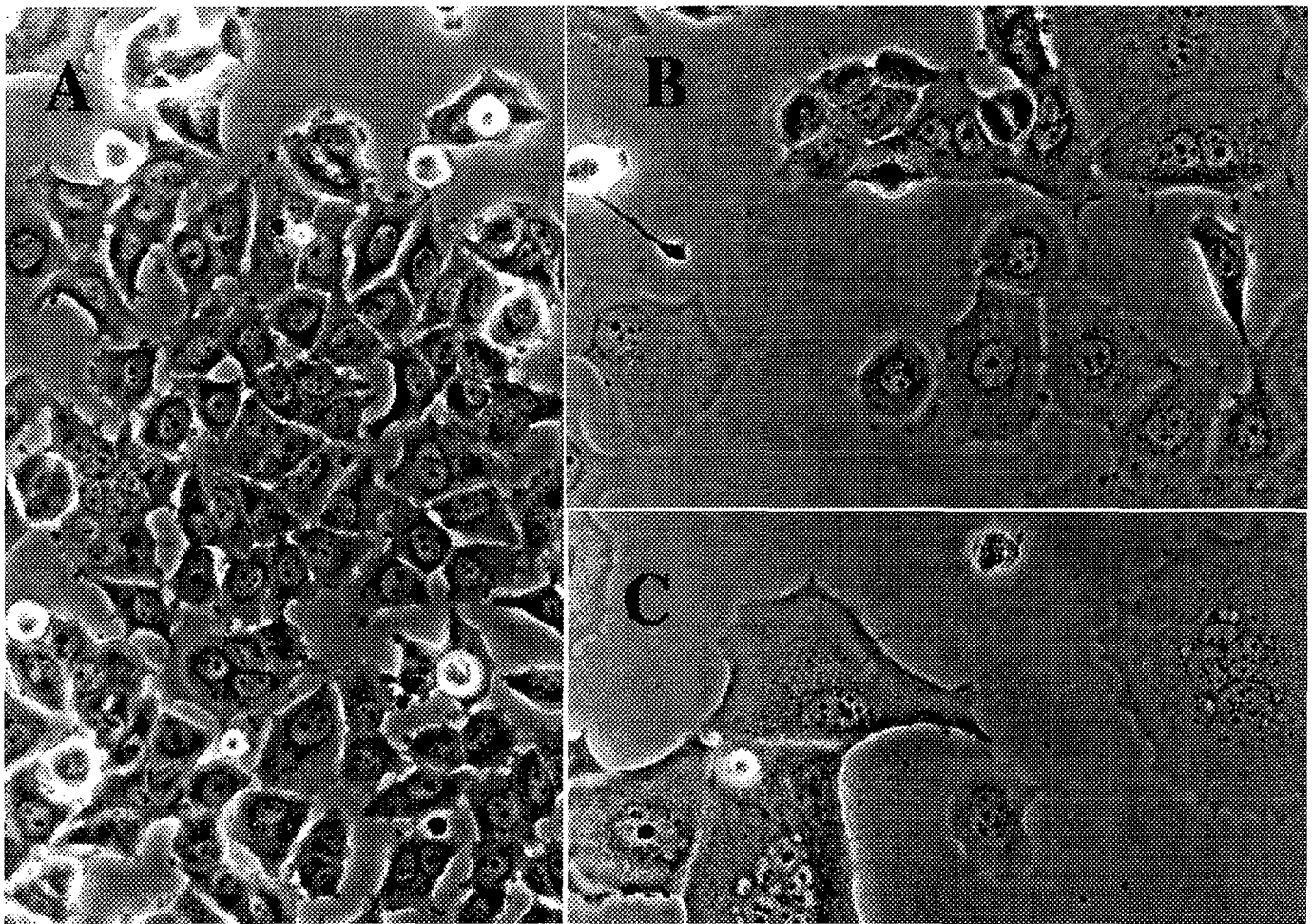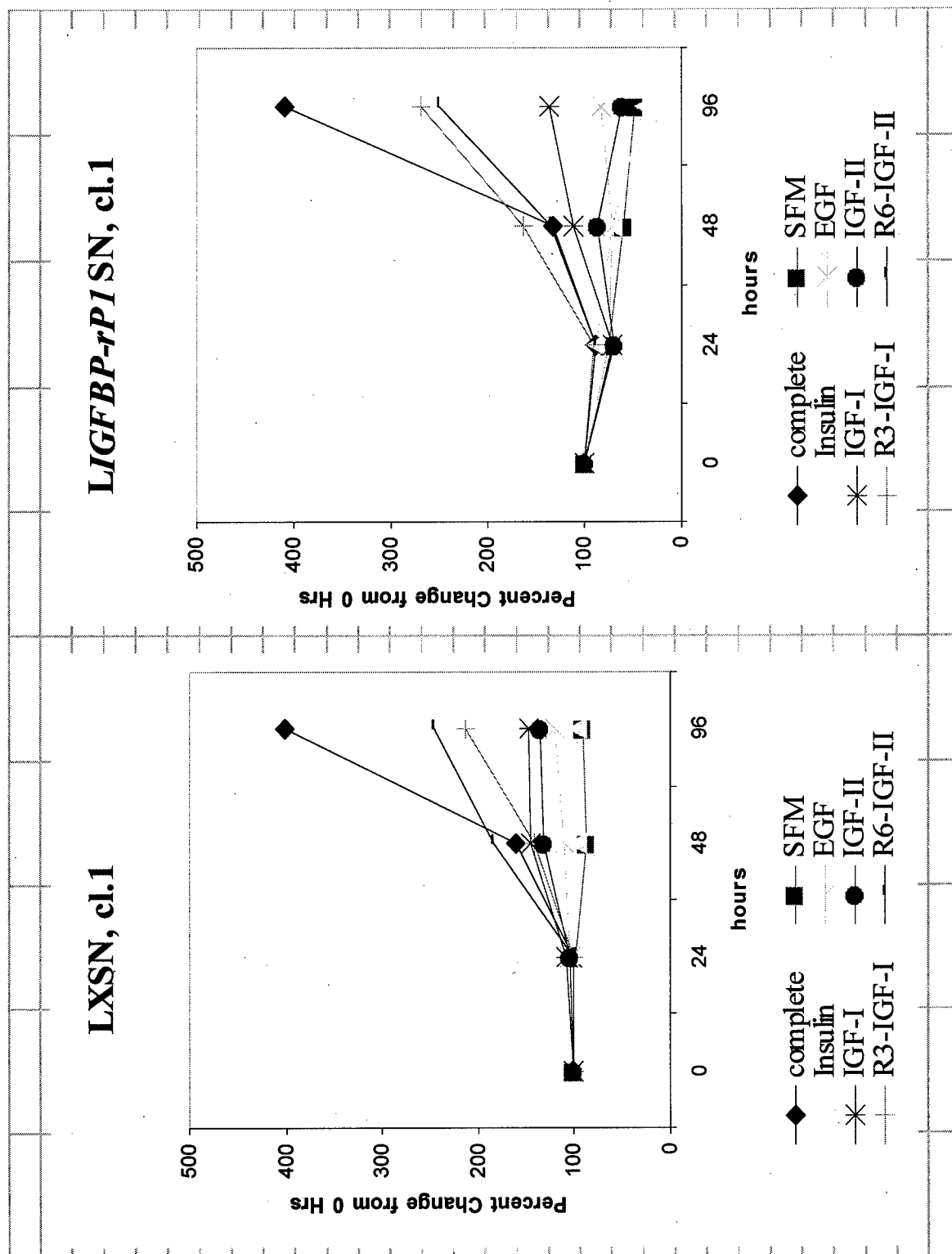
TABLE 1

Comparison of cell viability and cell cycle phases between MCF7 breast cancer cells transduced with LXSN and L*IGFBP-RP-1*SN.

Cell Line	%G1	%G2	%S	G2/G1	Live	Apoptotic	Debris
LXSN, T2	48.9 +/-1.27	17.5 +/-6.76	33.5 +/-6.65	1.9 +/-0.05	86.8 +/-0.85	8.7 +/-0.11	5.1 +/-1.08
L*IGFBP-RP-1*SN, T2	51.2 +/-2.75	18.2 +/-1.5	30.6 +/-1.61	1.9 +/-0.02	87.8 +/-0.90	7.3 +/-0.79	5.1 +/-0.19
	p=0.156	*p=0.452*	*p=0.222*	*p=0.291*	*p=0.021*	*p=0.054*	*p=0.492*
LXSN, cl.1	48.2 +/-1.18	20.8 +/-2.99	31.1 +/-2.42	1.9 +/- 0.03	89.2 +/-0.31	6.1 +/- 0.94	4.7 +/-0.50
L*IGFBP-RP-1*SN, cl.1	59.8 +/-2.71	17.2 +/-1.94	23.0 +/-1.21	1.8 +/-0.02	84.2 +/-1.35	7.1 +/-1.41	8.9 +/-1.85
	p=0.017	*p=0.107*	*p=0.009*	*p=0.011*	*p=0.017*	*p=0.253*	*p=0.045*
LXSN, cl.2	51.3 +/-3.04	15.7 +/-5.94	33.0 +/-5.33	1.9 +/-0.06	88.9 +/-0.50	6.7 +/-0.14	4.8 +/-0.22
L*IGFBP-RP-1*SN, cl.2	54.1 +/-3.09	18.7 +/-4.75	27.3 +/-1.65	1.9 +/-0.02	83.7 +/-0.56	8.8 +/-0.21	7.8 +/-0.60
	p=0.159	*p=0.047*	*p=0.059*	*p=0.17*	*p=0.005*	*p=0.003*	*p=0.008*
LXSN, cl.3	49.8 +/-0.90	18.5 +/-1.76	31.7 +/-1.51	1.9 +/-0.02	86.1 +/-1.89	10.9 +/-2.00	3.6 +/-0.08
L*IGFBP-RP-1*SN, cl.3	43.8 +/-2.18	27.5 +/-3.05	28.7 +/-1.07	1.9 +/-0.02	87.4 +/-0.22	7.5 +/-0.61	4.6 +/-0.77
	p=0.013	*p=0.003*	*p=0.005*	*p=0.003*	*p=0.165*	*p=0.075*	*p=0.089*

Note. Cell viability and cell cycle was analyzed by flow cytometry as described under Materials and Methods. Percentages are average of three samples. Student's *t* test was used to distinguish between paired populations.

TABLE 2

Comparison of apoptosis and senescence associated β-gal activity in MCF7 breast cancer cells transduced with LXSN and L*IGFBP-RP-1*SN.

	Percent Apoptotic	β-gal Activity
parental	1.5	2.2
LXSN, T2	1.6	2.3
L*IGFBP-RP-1*SN, T2	0.8	29.0
LXSN, cl.1	1.0	0.5
L*IGFBP-RP-1*SN, cl.1	0.7	85.2
LXSN, cl.2	1.0	0.3
L*IGFBP-RP-1*SN, cl.2	1.3	6.7
LXSN, cl.3	0.8	2.2
L*IGFBP-RP-1*SN, cl.3	0.5	87.1

Note. Nuclear fragmentation assay and senescence-associated β-galactosidase staining were performed as described under Material and Methods. 1000 cells were scored to determine percentages for nuclear fragmentation assay and 500 cells were counted to calculate percent β-galactosidase activity.

REPLY TO
ATTENTION OF:

MCMR-RMI-S   (70-1y)   23 Aug 01

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Encl   PHYLIS M. RINEHART
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